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Expression of membrane-bound human aminopeptidaseP as a soluble enzyme and an investigation into its efficacy towards offering protection against the toxicity of chemical warfare nerve agents

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14. ABSTRACT

In humans there are two forms of aminopeptidaseP (APP), a cytosolic and a membrane-bound (hmAPP). In this study, we produced an adenovirus containing the hmAPP gene without the membrane binding domain and containing a C-terminus 6 × histidine tag (Ad-shmAPP); this virus was used for expression of soluble hmAPP in HEK293A cells in vitro and in mice in vivo. The enzyme from HEK293A cells was purified and investigated for its ability to hydrolyze GD, GB, GF, GA, and VX. While unable to hydrolyze VX, shmAPP was capable of hydrolyzing each G agent, hydrolyzing GD more efficiently than any other G-type nerve agent with a catalytic efficiency of (5 ± 1) × 106 M-1 min-1. However, the stereochemical preference of shmAPP favors the hydrolysis of the non-toxic P (+) isomer(s) of each agent. We then evaluated whether mice containing elevated blood levels of shmAPP would be protected from the lethality of GD, GF and GA. Mice were infected with Ad-shmAPP and 5 days later were challenged subcutaneously with 1 x LD50 doses of GA, GF and GD. The survival rates of Ad-shmAPP-treated mice were not significantly different from those of mice treated with a control virus. These results suggest that the overexpression of wild-type shmAPP in mice failed to afford protection against nerve agent toxicity.

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Abstract

AminopeptidaseP (APP) is a ubiquitous enzyme found in a host of organisms, including bacteria, yeast and vertebrates. In humans there are two forms of APP, a cytosolic and a membrane-bound (hmAPP). In this study, we have produced an adenovirus containing the hmAPP gene without the membrane binding domain and containing a C-terminus 6 × histidine tag (Ad-shmAPP); this virus was used for expression of soluble hmAPP in HEK293A cells *in vitro* and in mice *in vivo*. The enzyme from HEK293A cells was purified and was investigated for its ability to hydrolyze soman (GD), sarin (GB), cyclo-sarin (GF), tabun (GA), and VX. While shmAPP was unable to hydrolyze VX, the enzyme was capable of hydrolyzing each G agent and was found to hydrolyze GD more efficiently than any other G-type nerve agent with a catalytic efficiency of $(5 \pm 1) \times 10^6$ M^{-1} min^{-1}. However, the stereochemical preference of shmAPP favors the hydrolysis of the non-toxic P (+) isomer(s) of each agent. We then evaluated whether mice containing elevated blood levels of shmAPP would be protected from the lethality of GD, GF and GA. Mice were infected with Ad-shmAPP and 5 days later, when the enzyme expression levels in mouse blood were highest, were challenged with 1 x LD_{50} doses of GA, GF and GD subcutaneously. The results show that the survival rates of Ad-shmAPP-treated mice were not significantly different from those of mice treated with a control virus. These results suggest that although wild-type shmAPP hydrolyzes G-type nerve agents *in vitro*, its overexpression in mice failed to afford protection against nerve agent toxicity.

1. Introduction

One of the highly investigated approaches to combat nerve agent exposure is the use of enzymes as bioscavengers to detoxify nerve agent prior to acetylcholinesterase (AChE) inhibition at neuromuscular junctions and nerve synapses [1, 2]. Native or recombinant human butyrylcholinesterase (BChE), a stoichiometric enzyme, has provided protection against nerve agent exposure in multiple animal models [3-5]. One of the disadvantages in using BChE as a medical countermeasure against nerve agents is that this enzyme is required in a large dose to afford protection. This is due to depletion of BChE upon inactivation of the nerve agent. It is estimated that a dose of 200 mg of the pure enzyme is necessary to protect a 70 kg individual against a 2 × LD_{50} dose of soman (GD) [6].

Catalytic enzymes are another class of bioscavengers that have the potential to hydrolyze nerve agent more effectively because of their ability to hydrolyze multiple equivalents of the nerve agent without becoming inactivated after hydrolysis. These properties offer catalytic bioscavengers inherent advantages over stoichiometric bioscavengers by having the potential to offer protection when present at significantly lower concentrations, and therefore requiring a lower administered dose of the prophylactic to achieve the same degree of protection as a stoichiometric bioscavenger [7]. Human liver prolidase, paraxonase-1, and organophosphate hydrolase are examples of catalytic bioscavengers under evaluation for their use as medical countermeasures against nerve agent exposure [8, 9].

Another potential candidate for use as a catalytic bioscavenger against nerve agents is human aminopeptidaseP (hAPP), which has high sequence homology to human liver prolidase [7,8]. It has also been shown that *E.coli* APP hydrolyzes several organophosphate derivatives of sarin (GB) [10]. The putative *in vivo* function of APP appears to be the cleavage of an *N*-terminal amino acid residue from peptides exhibiting a proline at P-1 residue such as bradykinin [11]. In humans, two forms of APP are present: a soluble cytosolic APP (hcAPP) and a membrane-bound APP (hmAPP) expressed by XPNPEP1 and XPNPEP2 genes respectively [12, 13]. HmAPP is a glycosylated, multimeric protein, which is anchored to the surface of the plasma membrane of endothelial and epithelial cells through a glycosylphosphatidylinositol (GPI) anchor. While hmAPP is found in many different tissues, a small quantity of the protein is also found in plasma due to the cleavage of the GPI anchor [14, 15]. A soluble form of hmAPP (shmAPP) was recently produced by adding a stop codon before the GPI linkage region [11] and was found to be stable and active against different bradykinins [11, 16]. In the present study, we investigated the ability of shmAPP to hydrolyze nerve agents, including GA, GB, GD, GF and VX, *in vitro* and to provide protection against nerve agent intoxication in mice *in vivo*.

2. Materials and Methods

2.1 Materials

Chromatography columns were purchased from GE Healthcare (Piscataway, NJ). Protein quantification reagent and M-PER extraction reagent were purchased from Pierce (Rockford, IL). Diisopropylfluorophosphate (DFP) and all other analytical chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Precast SDS-PAGE gels were purchased from BioRad Inc. (Hercules, CA). APP substrate, Lys(Nε-Abz)-Pro-Pro-pNA, was purchased from Bachem (Torrance, CA). Rabbit α-6 × His tag antibody was purchased from AbCam (Cambridge, MA). Nerve agents *O*-ethyl *N*,*N*-dimethylphosphoramidocyanidate (GA),

isopropoxymethylphosphoryl fluoride (GB), cyclohexyl methylphosphonofluoridate (GF), *O*-pinacolyl methyphosphonofluoridate (GD), and *O*-ethyl *S*-2-*N*,*N*-diisopropylaminoethyl methylphosphonothiolate (VX) were obtained from the U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. The purity of NA was >98.5% as determined by ^{31}P NMR.

2.2 APP plasmid engineering

A secreted form of membrane-bound hAPP (shmAPP) was engineered as previously described (7). Using PCR, a translational stop codon (TGA) was introduced at amino acid residue 658, replacing the Trp codon (TGG) immediately upstream of the hydrophobic peptide acting as the GPI-anchoring signal. A 6 × histidine tag was also introduced in frame immediately before the stop codon. The PCR product was initially cloned into the expression plasmid pCMV 6-Entry (Origene, Rockville, MD) and was fully sequenced to ensure authenticity of the shmAPP gene sequence. The shmAPP gene was then transferred into an adenoviral transfer vector (pENT-CMV, Welgen Inc., Worcester, MA) and was used to generate an adenovirus capable of expressing recombinant shmAPP (Ad-shmAPP) following standard protocols (Welgen Inc., Worcester, MA).

2.3 Cell culture and infection with Ad-shmAPP

HEK293A cells (1×10^6) were grown in 6-well plates in growth medium (DMEM containing 10% FBS, penicillin and streptomycin [50 units/mL], L-glutamine [2 mM]). After 24 hrs, the cells were infected with 5-100 viral particles (vp) per cell of Ad-shmAPP or 10 vp/cell of Ad-null (control virus) in 1.0 mL of 2% FBS in DMEM (infection medium), and incubated for 2 hrs at 37 °C. Then 1.0 mL of growth medium was added, and cells were incubated at 37 °C for 3 to 5 days. APP expression was determined by an APP activity assay [17] and was confirmed by Western blot using a rabbit α-6× histidine tag antibody.

2.4 Expression levels of shmAPP in culture medium vs cell lysate

Cells were infected with Ad-shmAPP (20 vp/cell), and 72 hours later the culture medium (2 mL) and cell pellet were collected. The cell pellet was lysed in 40 µL of M-PER reagent, and the lysate was collected following centrifugation at 14,000 g for 15 min. The expression levels of shmAPP in 5 µL of the cell lysate and 15 µL of the culture medium were determined by Western blot as described above.

2.5 APP activity assay

APP activity was recorded in 96-well format on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) spectrophotometer. To test for APP activity, 10 µL of media was added to 50 µL of reaction buffer (100 mM HEPES, 0.5 mM $MnCl_2$). Then 50 µL of 100 µM Lys(Nε-Abz)-Pro-Pro-pNA was added. The release of Lys(N^{ε}-Abz)-OH was monitored by the increase in fluorescence signal (ex:320, em:410). To relate the change in fluorescence to the concentration of expressed shmAPP in mouse plasma, a standard curve of Lys(Nε-Abz)-Pro-Pro-pNA hydrolysis was constructed using 0.02 – 0.12 µg of purified shmAPP. The concentration of expressed shmAPP in mouse plasma was then further confirmed by semi-quantitative Western blot as described above.

2.6 Large-scale expression and purification of shmAPP

HEK293A cells (1.0×10^7) were seeded in 150 cm^2 tissue culture dishes and allowed to attach to the tissue culture plastic for 16-24 hrs. The following day, cells were infected with 20 vp/cell of Ad-shmAPP in 10 mL of infection medium and incubated at 37 °C. Two hours later, 15 mL of the growth was added to each dish, cell cultures were returned to the incubator, and 5 days later medium was collected and centrifuged at 3000 x g for 15 min at 4 °C to remove the cellular debris.

Solid ammonium sulfate was added to the cell culture medium to raise its concentration from 0 to 50% and was subjected to centrifugation at 13000 x g for 30 min. Protein precipitate was discarded, and the ammonium sulfate concentration in the supernatant was further raised from 50% to 75%. After centrifugation at 13,000 g for 30 min, the 50-75% ammonium sulfate precipitate was collected and resuspended in 10 mL of 20 mM Tris-HCl, pH 8.0, containing 15 mM NaCl and 2 mM $MnCl_2$ and was buffer exchanged into buffer A (20 mM Tris-HCl, pH 8.0, containing 15 mM imidazole, 500 mM NaCl and 2 mM $MnCl_2$). The sample was then loaded onto a 5 mL pre-packed nickel-affinity resin column. The column was washed with 5 column volumes of buffer A, and bound proteins were eluted with 20 column volumes of a linear ascending gradient of buffer A containing 400 mM imidazole at a flow rate of 1 mL per min. Fractions containing APP activity were pooled, desalted using Amicon 10-kDa concentrators and visualized by SDS-PAGE to determine the purity of shmAPP.

2.7 Nerve agent hydrolysis

The hydrolysis assay for G-type nerve agents was performed as described [17]. Briefly, 0.027 nmol of purified shmAPP was incubated for 30 min in 100 mM MOPS, pH 8.0 and 2 mM $MnCl_2$. After incubation, a racemic sample of G agent (175 nmol of GA, 203 nmol of GB, 156 nmol or GD, or 158 nmol of GF) was added to start the reaction. At specific time intervals 50 µL aliquots were removed and inactivated through extraction with an equal volume of ethyl acetate containing 50 µM DFP as an internal standard. The organic layer (containing non-hydrolyzed nerve agent) was then removed and analyzed by gas chromatography/mass spectrometry (GC/MS) using an Agilent 6890 GC equipped with a 7890 MS detector [17, 18]. The separation of each stereoisomer was achieved using a Chiral $Dex^{(R)}$(TA) column (20M x 250 µm x 0.10 µm) (Advanced Separations Technologies Inc., Whippany, NJ). The residual amount of unhydrolyzed nerve agent in each sample was normalized by comparison to both the DFP internal standard and a standard calibration curve. The catalytic efficiency ($kcat/K_M$) for each agent was approximated by fitting the data to a one-phase exponential decay curve (GraphPad, version 5). The stereochemical preference of the enzyme toward the more toxic P(-)isomer was determined by the ratio of the apparent hydrolysis of the P (-) isomer to the P (+) isomer within the racemic mixture. The hydrolysis of VX was determined using a modified Ellman colorimetric assay as previously described [18].

2.8 In vivo expression of shmAPP

For these studies, Swiss-Webster mice (male, 20–25 g body weight) were housed at 20 °C and were provided food and water *ad libitum*. Mice (n=6) were injected with 2×10^{11} viral particles of Ad-shmAPP or Ad-null per mouse via the tail vein. Prior to adenovirus infection and daily for 7 days, 25 to 50 µL of blood was collected via saphenous vein puncture into heparin-coated tubes. The blood was centrifuged at 5000 g for 10 min to isolate plasma. The concentration of shmAPP in each mouse was determined by the APP activity assay as described

above. The change in fluorescence was used to determine the concentration of shmAPP by comparison to a standard curve constructed by recording the hydrolysis of Lys(Nε-Abz)-Pro-Pro-pNA by varying amounts of purified APP as described above and were confirmed by quantitative Western blot.

2.9 In vivo efficacy of Ad-shmAPP

Ad-shmAPP or Ad-null viruses were injected into mice as described above. Five days later, 20 μL of blood was collected, and the animals were challenged by subcutaneous injection of a 1×LD_{50} dose of GA (220 μg/kg), GD (110μg/kg) or GF (110 μg/kg). Mice were observed for mortality for 1 hr after exposure to the agent. Surviving animals, if any in each group, were again challenged with a second 1 × LD_{50} dose of the respective nerve agent and were observed for mortality.

3. Results and Discussion

3.1 Expression, purification, and characterization of shmAPP.

The ability of Ad-shmAPP to express a secreted form of recombinant shmAPP was investigated in HEK293A cells. Cells were infected with varying concentrations of Ad-shmAPP (5 vp to 100 vp/cell); culture medium was collected after 48 hours, concentrated and assayed for the presence of recombinant shmAPP by Western blot using an antibody against 6 × histidine tag. As shown in Figure 1A the presence of a ~75kDa protein similar to the molecular weight expected for full-length shmAPP was detected in the culture medium of cells infected with 10 vp/cell or higher but not in Ad-null-treated cells. Next, we determined whether the Ad-shmAPP expressed protein is biologically active by measuring its ability to hydrolyze a synthetic fluorescence substrate, Lys(Nε-Abz)-Pro-Pro-pNA. HEK293A cells were infected with 20 vp/cell of Ad-shmAPP; medium was collected on days 3, 4, and 5 and assayed for substrate hydrolysis. As shown in Figure 1B, APP activity was readily detected in the culture medium of Ad-shmAPP-infected cells but not in the medium from Ad-null-infected cells. Enzyme activity was highest in day 5 media compared to media on days 3 and 4. The presence of shmAPP in each media sample was also confirmed by Western blot (Figure 1B insert). These results suggest that Ad-shmAPP was biologically active and transduced the expression of soluble, full-length and active recombinant shmAPP *in vitro*.

One of the aims of our investigation was to produce mostly soluble hmAPP *in vitro* in HEK293A cells such that purification and characterization of the enzyme with regard to its ability to hydrolyze nerve agents will be feasible. To test whether the Ad-shmAPP-expressed enzyme is mostly secreted, HEK293A cells were infected with 20 vp/cell, and 3 days later culture medium and cell lysate samples were collected and processed to determine the concentration of shmAPP by Western blot. This experiment was done quantitatively to assess the distribution of the expressed shmAPP in the media versus in the cell extract. As shown in Figure 1C, 15 μL of the cell culture medium contained ~ 3-fold more shmAPP as compared to 5 μL of the cell lysate sample. Extrapolating the 75 kDa shmAPP band intensity to the total amount of the culture medium (2 mL) and lysate sample (40 μL) indicates that >90% of the enzyme was present in the culture medium as compared to < 10% present in the cell lysate. These data suggest that most of the shmAPP expressed in HEK293A cells was secreted into the culture medium.

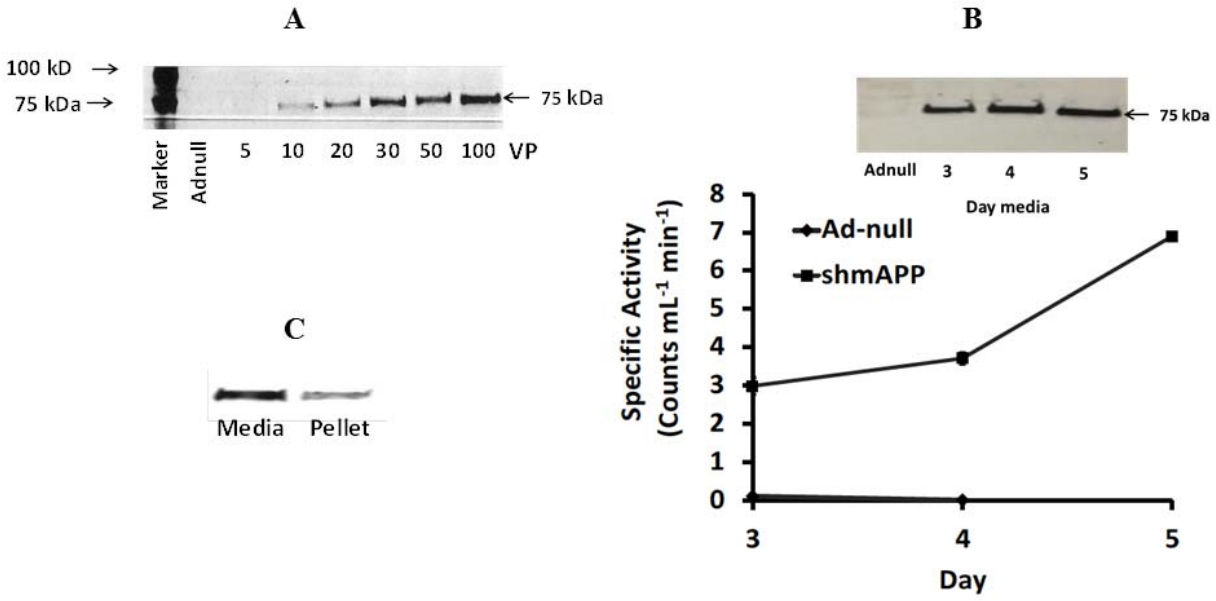

Figure 1: A. Ad-shmAPP was expressed in HEK 293A cells infected with varying amounts of viral particles from 5 vp to 100 vp/cell. The expression was monitored after 2 days post-infection. The expression of shmAPP in media was visualized by Western blot using a rabbit α-6×His tag antibody. B. Media from HEK 293A cells infected with 20 vp of either Ad-shmAPP or Ad-null were collected on days 3, 4 and 5 post-infection and assayed for APP activity using the fluorescent substrate Lys(Nε-Abz)-Pro-Pro-pNA (ex:320, em:410). The time-dependent expression of APP was also visualized by Western blot as described above, which showed a pattern of expression similar to that displayed by the APP activity assay. C. The expression of shmAPP was quantitated inside HEK293A cells compared to the amount secreted into the media as described in Materials and Methods.

Culture medium from HEK293A cells infected with 20 vp/cell was collected on day 5 and was used for purification of recombinant shmAPP by ammonium sulfate fractionation followed by affinity chromatography involving nickel-affinity resin. The activity of shmAPP in the ammonium-sulfate precipitates and affinity column fractions was monitored by the APP hydrolysis assay. The protein precipitant resulting from increasing the ammonium sulfate concentration in the medium from 50 - 75% contained most of the APP activity. Further purification of the enzyme was obtained by subjecting the re-suspended precipitant through nickel-affinity chromatography (Figure 2). The result shows that shmAPP was eluted as a single peak encompassing fractions 45 to 60 using a 10 - 400 mM imidazole gradient (Figure 2A). These fractions were pooled, concentrated and visualized by SDS-PAGE followed by coomassie staining to determine the purity of shmAPP. As shown in Figure 2B, shmAPP appeared as a single band with an approximate molecular weight of 75 kDa. Employing this purification scheme, 400 μg of purified shmAPP was obtained per one liter of culture medium.

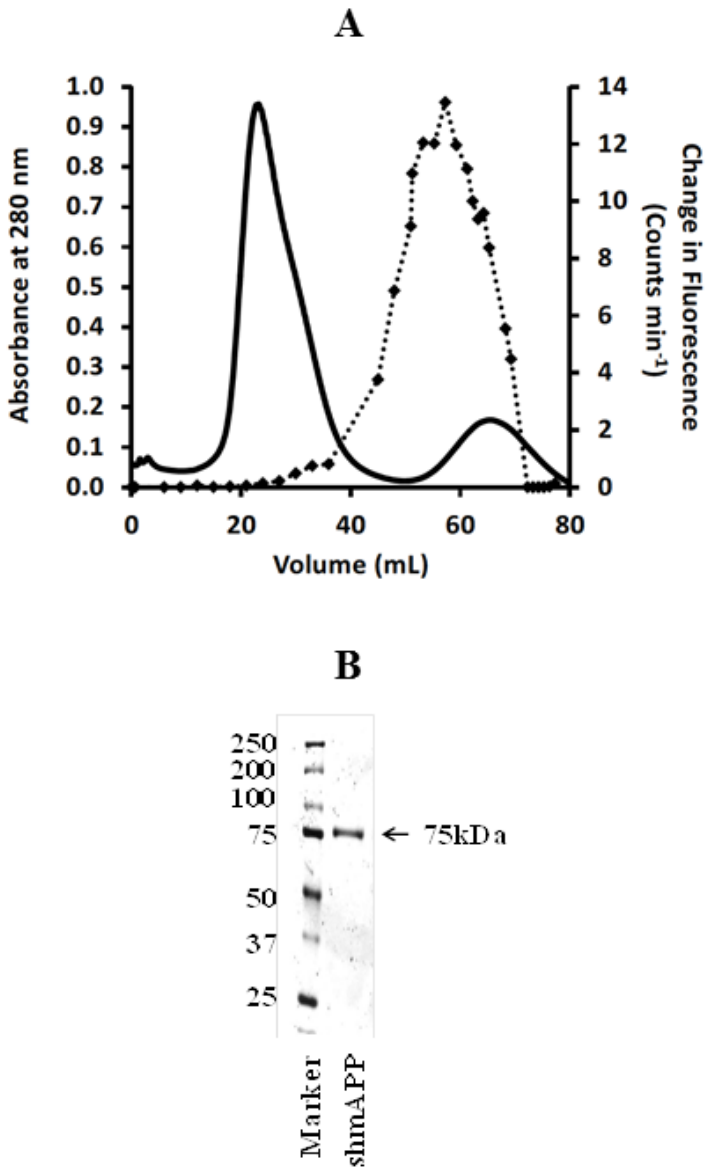

Figure 2: Purification of shmAPP. A. To purify shmAPP, Ni-NTA affinity chromatography was used. Bound shmAPP was eluted with a linear gradient of 15 – 400 mM imidazole at a flow rate of 0.5 mL per min. The solid line is the total protein absorbance at 280 nm, while the dotted line represents APP activity determined by measuring the hydrolysis of APP substrate Lys(Nε-Abz)-Pro-Pro-pNA in fluorescence signal counts per minute. B. Fractions containing APP activity were analyzed by SDS-PAGE, and proteins were stained by Coomassie staining, which showed that the 75kDa APP protein was purified to homogeneity.

3.2 In vitro hydrolysis of nerve agents by shmAPP

Purified shmAPP was evaluated for its ability to hydrolyze a variety of G agents, including GA, GB, GD, and GF, and V agents, to include VX. Hydrolysis of G agents was measured using GC/MS to quantitate the residual amount of each agent stereoisomer at fixed time intervals. As shown in Table 1 and Figure 3, purified shmAPP was able to hydrolyze all the G agents, but failed to hydrolyze VX at a detectable rate (data not shown). The catalytic efficiencies for G agent hydrolysis ranged from 5×10^6 M^{-1} min^{-1} for GD to 4×10^5 M^{-1} min^{-1} for GB (Table 1). In all cases, the more toxic P (-) isomer was hydrolyzed less efficiently than the less toxic P (+) isomer. Taken together, these results suggest that shmAPP is capable of hydrolyzing G agents but prefers the hydrolysis of the less toxic P (+) isomers over the more toxic P (-) isomers.

Agent	**P(-) Isomer Apparent k_{cat}/K_m ($\times 10^5$ M^{-1} min^{-1})**	**P(+) Isomer Apparent k_{cat}/K_m ($\times 10^5$ M^{-1} min^{-1})**	**Racemic k_{cat}/K_m ($\times 10^5$ M^{-1} min^{-1})**	**Ratio of P (-)/P (+)**
GA	**1.7 ± 0.1**	**5 ±1**	**7 ± 1**	**0.32**
GB	**2.1 ± 0.3**	**4 ± 1**	**6 ±1**	**0.57**
GD	**4.3 ± 0.4**	**50 ± 10**	**60 ± 10**	**0.08**
GF	**3.1 ± 0.2**	**18 ± 9**	**20 ± 9**	**0.17**
VX	**Not Detected**	**Not Detected**	**Not Detected**	**Not Detected**

Table 1: Catalytic parameters (apparent kcat/Km) of purified shmAPP enzyme against G-type nerve agents. Purified shmAPP was evaluated *in vitro* for its ability to hydrolyze the nerve agents GA, GB, GD and GF. Activity against VX was not detected.

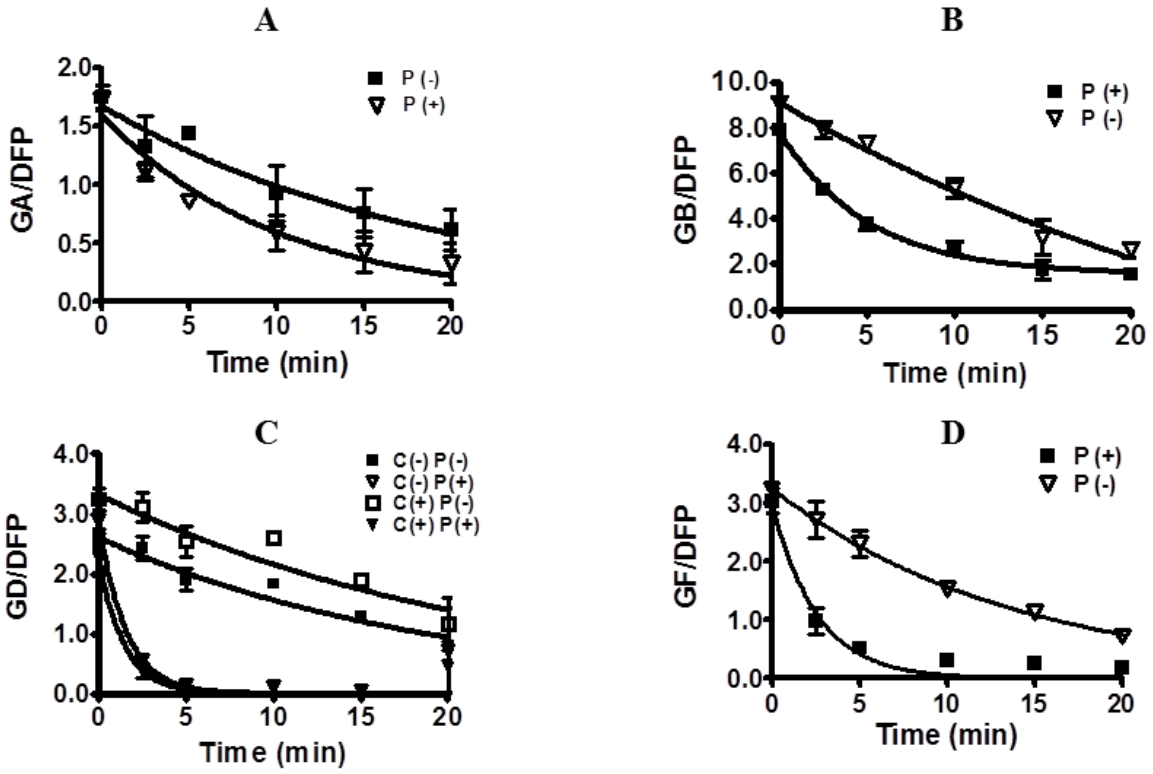

Figure 3: Hydrolysis of G agents by shmAPP. The hydrolysis of GA (A), GB (B), GD (C) and GF (D) was determined using a modified GC/MS detection assay. The rate of depletion of each G agent by shmAPP was determined by fitting the integrated peak area of each isomer at fixed time points to a single phase exponential decay. The concentration of shmAPP in each experiment was 242 nM.

3.3 In vivo expression of shmAPP

One of the challenging aspects of determining the efficacy of an enzyme against nerve agent toxicity is to achieve high levels of the enzyme expression for several days in animal blood. In previous studies, we have shown that the adenovirus-expression system, which we employed here for the expression of shmAPP, is able to induce the expression of human and mouse BChE, and wild-type human PON1 at very high levels for 4 to 7 days in mouse blood following a single tail vein injection of the respective virus [5, 19-21]. Mice containing elevated blood levels of BChE were protected from the lethality of echothiophate, GD, GB, GA, and GD [22-25], while mice containing elevated blood levels of wild-type human PON1 were protected from diazoxon but not from G agents or VX [26]. Therefore, we tested the ability of Ad-shmAPP to induce the expression of recombinant shmAPP in mouse plasma. Mice (n=6) were injected with Ad-shmAPP, and plasma samples on days 3 to 7 after virus infection were assayed for shmAPP levels by the APP activity assay. As shown in Figure 4A, APP activity was detected on day 3, reached peak levels on day 5, and gradually decreased on days 6 and 7. On day 7, the APP activity levels were still well above the baseline levels in control virus-injected animals. Western blot of plasma from Ad-shmAPP-injected mice confirmed the presence of ~ 75 kDa shmAPP protein (Figure 4B), and the band intensity correlated with the activity profile (Figure 4A). These results suggest that a single tail vein injection of the virus transduced the expression of persistent and high level expression of functionally active shmAPP in mouse blood. The

enzyme expression persisted for at least 5 days at levels well above the baseline established by the control animals.

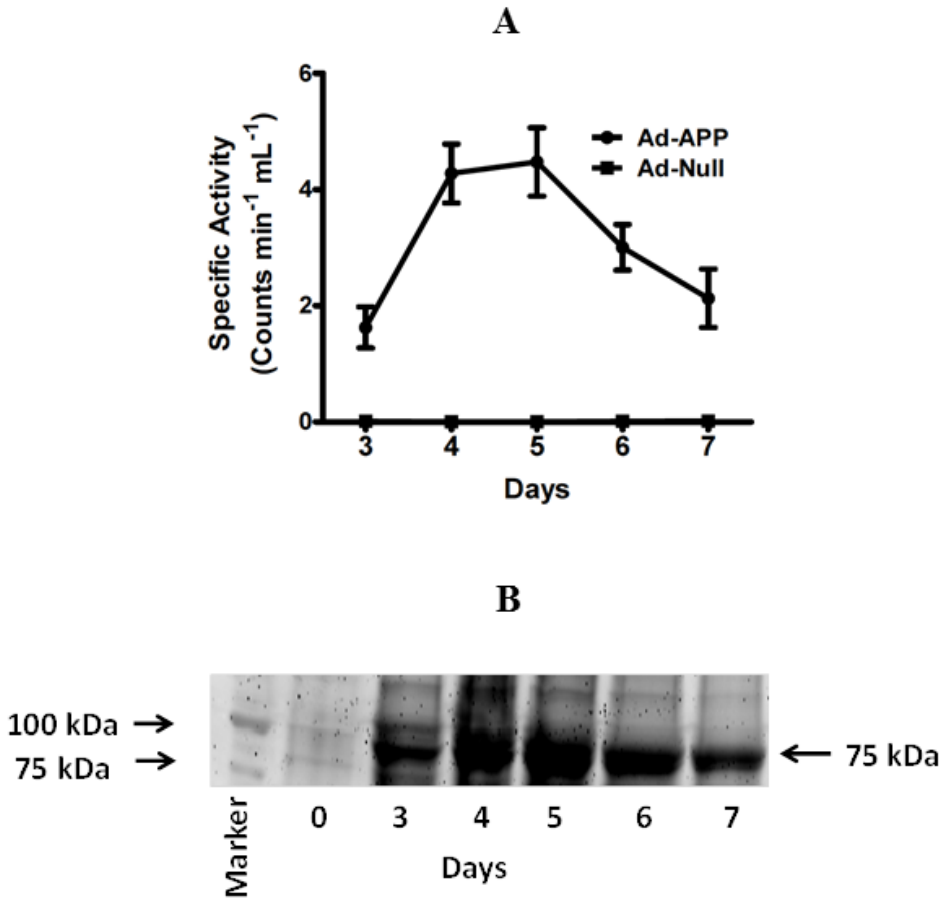

Figure 4: Expression of shmAPP in mice plasma. A. Swiss Webster male mice were injected with Ad-shmAPP (2 × 1011 viral particles per mouse) via the tail, and expression of recombinant shmAPP was determined in plasma over a 7-day period using the fluorescent substrate Lys(Nε-Abz)-Pro-Pro-pNA. B. Quantitative Western blot of mouse plasma showing the 75kDa protein band visualized as described above.

3.4 In vivo evaluation of the protective efficacy of Ad-shmAPP against G-type nerve agents

The protection offered by Ad-shmAPP against the toxicity of G-type nerve agents was evaluated in mice. Protection against VX was not evaluated since purified shmAPP failed to hydrolyze VX *in vitro*. Groups of mice (n=4 - 6) were injected with Ad-null or Ad-shmAPP, and on day 5, the animals were challenged with a 1 × LD_{50} dose of GA, GD, and/or GF. If animals survived, they were challenged with another 1 × LD_{50} dose of the respective agent 1 hour after the first challenge. The nerve agent challenge was performed on day 5 since maximum enzyme expression was achieved on this day (Figure 4A and 4B). For the GD challenge, 25% of both control and Ad-shmAPP-treated mice survived the first 1 × LD_{50} dose, but all of the animals died following the second 1 × LD_{50} challenge. A survival rate of 100% was observed for both Ad-null and Ad-shmAPP groups with the first 1 × LD_{50} of GA and GF challenge, but all of the mice died after the second 1 × LD_{50} challenge (Table 2). Blood enzyme levels prior to agent challenge

in Ad-shmAPP groups ranged from 0.56 – 1.8 mg/ml for GA, 0.11 – 0.79 mg/ml for GD and 0.27 – 1.2 mg/ml for GF (Table 2). This corresponds to molar equivalents of nerve agent administered ranging from 0.96 – 3.1 for GA, 0.97– 7.0 for GD and 1.3 – 5.6 for GF per equivalent of enzyme at the time of challenge. Thus, in some animals the enzyme was present in excess of the agent, but the animals were not protected.

Agent	Number of animals challenged	Virus injected	shmAPP concentration range (mg/mL)	Molar equivalents of agent per challenge ($1 \times LD_{50}$)	Percent survival 1^{st} ($1 \times LD_{50}$) challenge	Percent survival 2^{nd} ($1 \times LD_{50}$) challenge
GA	6	Ad-null	ND	ND	100	0
	5	Ad-shmAPP	0.56 – 1.8	0.96 – 3.1	100	0
GF	6	Ad-null	ND	ND	100	0
	4	Ad-shmAPP	0.27 – 1.2	1.3 – 5.6	100	0
GD	4	Ad-null	ND	ND	25	0
	4	Ad-shmAPP	0.11 – 0.79	0.97 – 7.0	25	0

Table 2: *In vivo* efficacy of APP against nerve agents in mice. Mice were infected through tail vein injections with Ad-null and Ad-shmAPP. On day 5 post-infection 20 µL of blood was collected prior to nerve agent challenge, and the concentration of shmAPP was determined by converting the hydrolysis of Lys(Nε-Abz)-Pro-Pro-pNA into APP concentration by interpolation from a standard curve. Each group of animals was challenged with a $1 \times LD_{50}$ dose of GA, GF or GD, and mortality was scored after 1 hour, after which time a second $1 \times LD_{50}$ dose of GA, GF and GD was administered to the surviving animals. Cumulative survival of both $1 \times LD_{50}$ doses, if any, was scored after 24 hours.

Taken together, the above studies reveal that wild-type shmAPP hydrolyzes G-type nerve agents *in vitro* but does not afford protection in our mouse model *in vivo*. Similar observations have been made for a number of candidate catalytic enzymes to include human liver prolidase (27), SMP30 [28], and human paraoxonase-1 [29-31]. A common property among all these enzymes was that they all favor the hydrolysis of the non-toxic P (+) isomer over the P (-) isomer [17,31]. Recently, Worek et al. reported that the PON1 mutant II-G1 favored the hydrolysis of the P (-) isomer, and its presence in blood circulation protected red blood cell and brain AChE and prolonged the survival rate of guinea pigs from GF toxicity [31]. The overall catalytic efficiency of II-G1 reported by Worek against GF was 8.4×10^7 M^{-1} min^{-1} and 6.4×10^7 M^{-1} min^{-1} for GD. Recently, we tested the ability the PON1 mutant I-F11 to offer 24 hr protection against GD-induced lethality in mice (Mata et al., Unpublished data). We found that this variant of PON1, when expressed in mouse blood, has an overall racemic catalytic efficiency against GD

that is somewhat similar to that of shmAPP. However, I-F11 greatly differs from shmAPP with respect to its stereochemical preference: I-F11 prefers the hydrolysis of the toxic P (-) isomer, whereas shmAPP prefers the hydrolysis of the non-toxic P (+) isomer. The adenovirus-mediated expression of I-F11 protected the mice against super-stoichiometric doses of GD even when the agent was in excess by several folds (Mata et al., Unpublished data). Based on these studies we propose that, while the apparent racemic k_{cat}/K_m of shmAPP against GD and GF appears to be high enough to offer protection against GD and GF *in vivo*, the fact that shmAPP failed to offer protection is due to insufficient catalytic efficiency against the more toxic P (-). These studies also suggest that shmAPP may offer protection against G agents provided its stereochemical preference can be reversed towards the P (-) isomer as was performed for PON1.

3.5 Conclusions

A soluble form of membrane-bound human aminopeptidaseP (shmAPP) was expressed and purified to homogeneity. The enzyme hydrolyzed G-type nerve agents including GA, GB, GD and GF *in vitro*. Fastest rate of hydrolysis was observed for GD followed by GF, GA, and GB. In all cases, the more toxic P (-) isomer was hydrolyzed less efficiently than the less toxic P (+) isomer. Its over-expression in mouse blood failed to afford protection against GD, GF, and GA *in vivo*.

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